AN INVESTIGATION INTO THE USE OF CARDIOLIPIN ANTIGENS*

I. ANTICOMPLEMENTARY ACTION OF CARDIOLIPIN

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INTRODUCTION

Ever since the discovery of cardiolipin by Pangborn (1941), much interest has been aroused by the use of this reagent in the sero-diagnosis of syphilis. In 1947, cardiolipin Wassermann antigen was tested, and the hope arose that it might be more sensitive than the ox-heart extract antigens employed in these laboratories (Price and Wilkinson, 1950). It was therefore decided to investigate the new reagent in detail in order to adapt it to the Whitechapel Wassermann technique (Price, 1950b). The primary object, therefore, of this investigation has been to ascertain the antigen formula of the three constituents, cardiolipin, lecithin, and cholesterol, which would work to the best technical advantage.

In the examination of any antigen for use in a complement-fixation test, two essential factors must be considered :

(1) the anticomplementary action of the antigen,
(2) its ability to fix antibodies (in this case reagin).

Method

The method of approach was to ascertain first the anticomplementary action, and then the ability to fix complement of each of the components of the antigen, individually, severally, and collectively. The techniques employed, such as the evaluation of complement doses used, the determination of the optimal titres of antigens, and quantitative testing procedures have already been described in a series of articles (Price and Wilkinson, 1947 ; Price 1949, 1950a, b) on the Whitechapel Wassermann technique.

It should be pointed out that the constituents of the cardiolipin Wassermann antigen are kept in alcoholic solutions and are made up at the strength indicated as a suspension in normal saline (0·9 per cent.). It will be noted that a saline suspension of 1 in 200 is frequently employed in the following investigations. This figure was chosen as approximating to the saline suspension titre of cardiolipin antigen, which had been found, in many instances, to give the most sensitive reactions.

The contents of the tubes in all experiments were :

(1) 1 volume of the appropriate serum, neat or diluted, or 1 volume of saline,
(2) 1 volume of the appropriate dilution of complement,
(3) 1 volume of the appropriate reagent or mixture of reagents to be tested.

The incubation times used were 30 min. at room temperature and 30 min. at 37° C., and the readings were made 30 min. after the sensitized red cells had been added and the mixtures kept at 37° C. The notation used is as follows :

\+ = no haemolysis
± = 50 per cent. haemolysis
± = 75 per cent. haemolysis
− = 100 per cent. haemolysis

PART 1—ANTICOMPLEMENTARY ACTION OF CARDIOLIPIN

Cardiolipin was tested using a range of from 0·6 to 0·004 per cent., each strength being mixed with normal saline at 1 in 50 and at 1 in 200. In the presence of normal serum, none of these mixtures showed any anticomplementary action. In the absence of normal serum, no anticomplementary action was evident when 1 in 200 saline suspensions were tested. If suspensions of 1 in 50 were employed, anticomplementary action was noted when the strength of the cardiolipin was 0·3 per cent. or more.

When mixtures of cardiolipin and lecithin were tested, no anticomplementary reaction was noted either in the presence or in the absence of normal serum. The cardiolipin ranges used were from 0·15 to 0·0125 per cent., and lecithin was added in constant amounts of 1·7 per cent. in one series, and 0·17 per cent. in the other. The mixtures were suspended in 1 in 200 of normal saline.

On testing mixtures of cardiolipin and cholesterol, a different picture was obtained. Three cardiolipin ranges from 0·15 to 0·006 per cent. were employed.

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To each unit of these cardiolipin ranges a constant amount of cholesterol was added; one range received 0·25 per cent., the second 0·5 per cent., and the third 1 per cent. Suspensions of these mixtures in saline were made at a strength of 1 in 200 and their anticomplementary powers were estimated. The results are given in Table I.

TABLE I

ANTICOMPLEMENTARY EFFECT OF INCREASING AMOUNTS OF CARDIOLIPIN IN CARDIOLIPIN-CHOLESTEROL MIXTURES

Cardiolipin (per cent)	Complement Dilutions					
	In absence of normal serum			In presence of normal serum		
	10	20	30	40	50	60
0·15	−	+	+	+	+	+
0·075	−	+	+	+	+	+
0·05	−	+	+	+	+	+
0·025	−	+	+	+	+	+
0·0125	−				+	+
0·006	−	−	−	−	±	+
Complement plus normal serum	−	−	−	−	±	+

Cholesterol constant (per cent) { —— 0·25 ---- 0·5 —○— 1·0

It will be seen that when these mixtures are tested in the absence of normal serum, the anticomplementary action becomes greater as the cardiolipin dose is increased until a strength of 0·025 per cent. is reached; above this point no further change was found. An increase in the cholesterol content from 0·25 to 0·5 per cent. enhances this anticomplementary action, but this is not affected by a further increase of this reagent to 1 per cent.

If the above mixtures are tested in the presence of normal serum, a difference in the reactions occurs. Table I shows that the presence of normal serum protects the complement from the destructive effects of the cardiolipin-cholesterol mixtures. The degree of protection depends on the amount of cholesterol present in the mixture. Thus, in the mixtures containing 0·25 per cent. cholesterol, protection is complete, but when 0·5 and 1 per cent. are employed a small but equal degree of anticomplementary action is discernible.

An examination of the anticomplementary powers of the three components of cardiolipin antigen when mixed together was next undertaken. The lecithin and cholesterol contents of the mixtures were kept constant at 0·05 and 0·5 per cent. respectively. Cardiolipin was added to these in varying strengths, starting at 0·0075 per cent. and working up to 0·15 per cent., and the anticomplementary titres of the various antigens were estimated.

Table II shows that no anticomplementary action was demonstrated in the *presence* of normal serum, whatever the strength of cardiolipin used in the mixtures. In the *absence* of normal serum, cardiolipin mixtures of 0·15 and 0·075 per cent. with lecithin 0·05 per cent. and cholesterol 0·5 per cent. gave anticomplementary titres at values of 1 in 320 and 1 in 160 respectively. Mixtures in normal saline above these dilutions were not anticomplementary.

TABLE II

ANTICOMPLEMENTARY EFFECT OF INCREASING AMOUNTS OF CARDIOLIPIN IN CARDIOLIPIN ANTIGENS

Antigen Contents (percent.)			Optimum Titre of Antigen	*AC Titre in absence of Normal Serum	*AC Titre in presence of Normal Serum	Ratios of Cardiolipin : Lecithin : Cholesterol
Cardiolipin	Lecithin	Cholesterol				
0·15	0·05	0·5	1/180	1/320	Nil	3 : 1 : 10
0·075	0·05	0·5	1/480	1/160	Nil	1·5 : 1 : 10
0·05	0·05	0·5	1/240	Nil	Nil	1·0 : 1 : 10
0·025	0·05	0·5	1/180	Nil	Nil	0·5 : 1 : 10
0·015	0·05	0·5	1/100	Nil	Nil	0·3 : 1 : 10
0·0075	0·05	0·5	1/60	Nil	Nil	0·15 : 1 : 10

*Anticomplementary titre may be defined as that suspension of antigen in the *greatest* amount of normal saline which will reveal anticomplementary action.

Table II shows a similar anticomplementary pattern to that obtained when cardiolipin and cholesterol were tested in the absence of normal serum. Under those conditions it was shown (see Table I) that, within limits, the more cardiolipin present in the mixture, the greater was the anticomplementary titre.

However, the presence of lecithin reduces the anticomplementary properties due to increased doses of cardiolipin. Thus, when cardiolipin is mixed with cholesterol and lecithin and tested in the *absence* of normal serum, no anticomplementary property is evoked until the strength of 0·075 per cent. cardiolipin is reached, whereas with a mixture of cardiolipin and a similar dose of cholesterol (0·5 per cent.) but no lecithin, some anticomplementary properties are evident even at the very much lower cardiolipin strength of 0·006 per cent. (see Table I).

From the preceding investigations, the following conclusions can be drawn:

(1) When suspensions of 0·6 per cent. or less of cardiolipin in 1 in 200 of normal saline are tested, no anticomplementary properties are exhibited.

(2) When suspensions of more than 0·3 per cent. cardiolipin in 1 in 50 normal saline are tested in the

absence of normal serum, anticomplementary action can be demonstrated.

(3) When mixtures of cardiolipin and lecithin are tested within the limits described, no anticomplementary action is apparent.

(4) Mixtures of cardiolipin and a constant amount of cholesterol are anticomplementary in the absence of normal serum. This property is increased *pari passu* with the amount of cardiolipin up to a strength of 0·025 per cent. Thereafter the pattern of the reaction remains constant (see Table I).

(5) In the presence of normal serum, however, the anticomplementary action of mixtures of cardiolipin and cholesterol is diminished, and in the case of mixtures containing 0·25 per cent. cholesterol is abolished.

(6) If cardiolipin Wassermann antigens containing increasing amounts of cardiolipin but constant amounts of lecithin (0·05 per cent.) and cholesterol (0·5 per cent.) are tested, the lecithin present completely protects the complement until the antigen contains between 0·05 and 0·075 per cent. of cardiolipin (see Table II).

(7) If cardiolipin-cholesterol mixtures or cardiolipin Wassermann antigens are tested in the presence of normal serum, protection of the complement can be demonstrated (see Tables I and II).

It would seem, therefore, that the anticomplementary action of a cardiolipin Wassermann antigen is due to the presence of cardiolipin and cholesterol. When either of these components is increased in amount, so, within limits, does this property become greater. On the other hand, the presence of lecithin diminishes the anticomplementary action of the cardiolipin and cholesterol. Finally, normal serum neutralizes the anticomplementary power of these antigens.

ANTICOMPLEMENTARY ACTION OF LECITHIN.—The lecithin component of cardiolipin Wassermann antigen was then tested for its anticomplementary property. A range was used varying from 3·4 to 0·05 per cent. in saline suspensions of 1 in 25 and 1 in 100. On testing, none of these suspensions exhibited any anticomplementary action.

When mixtures of lecithin and cardiolipin were tested, no anticomplementary properties were discovered. The lecithin ranges were from 1·7 to 0·0125 per cent. To units of two sets of these ranges were added constant amounts of 0·15 and 0·05 per cent. cardiolipin respectively. The mixtures were then suspended 1 part in 200 of saline before examination.

Mixtures of lecithin and cholesterol were then tested, the ranges of lecithin being from 1·7 to 0·125 per cent. To the units of two sets of these ranges were added constant amounts of 1 and 0·5 per cent. cholesterol respectively. Suspensions of the mixtures were made 1 part in 200 of saline and tested for anticomplementary properties. None were demonstrated.

Finally, cardiolipin Wassermann antigens which contained varying amounts of lecithin were tested for anticomplementary action.

Table III shows that none of the antigens showed any anticomplementary power when tested in the presence of normal serum.

When tested in the absence of normal serum, the first three groups (each group containing varying amounts of lecithin but constant amounts of cardiolipin and cholesterol) evinced no anticomple-

TABLE III

ANTICOMPLEMENTARY EFFECT OF INCREASING AMOUNTS OF LECITHIN IN CARDIOLIPIN ANTIGENS

Group	Antigen Contents (percent.)			Optimum Titre of Antigen	AC Titre in absence of Normal Serum	AC Titre in presence of Normal Serum	Approximate Ratios of Lecithin : Cardiolipin : Cholesterol
	Lecithin	Cardiolipin	Cholesterol				
1	0·8	0·15	0·5	1/2560	Nil	Nil	7 : 1 : 4
	0·4	0·15	0·5	1/2560	Nil	Nil	3 : 1 : 4
	0·2	0·15	0·5	1/640	*Nil*	Nil	*1·5 : 1 : 4*
	0·15	0·15	0·5	1/640	1/40	Nil	1 : 1 : 4
	0·08	0·15	0·5	1/240	1/80	Nil	0·5 : 1 : 4
	0·04	0·15	0·5	1/180	1/80	Nil	0·25 : 1 : 4
	0·02	0·15	0·5	1/160	1/2560	Nil	0·125 : 1 : 4
2	0·8	0·075	0·5	1/480	Nil	Nil	10 : 1 : 6
	0·2	0·075	0·5	1/480	*Nil*	Nil	*3 : 1 : 6*
	0·08	0·075	0·5	1/480	1/40	Nil	1 : 1 : 6
	0·04	0·075	0·5	1/480	1/160	Nil	0·5 : 1 : 6
	0·01	0·075	0·5	1/170	1/640	Nil	0·17 : 1 : 6
3	*0·1*	0·06	0·5	1/360	*Nil*	Nil	*2 : 1 : 10*
	0·06	0·06	0·5	1/340	1/20	Nil	1 : 1 : 10
	0·03	0·06	0·5	1/50	1/160	Nil	0·5 : 1: 10
	0·015	0·06	0·5	1/50	1/320	Nil	0·25 : 1 : 10
4	0·5	0·05	0·5	1/480	Nil	Nil	10 : 1 : 10
	0·25	0·05	0·5	1/480	Nil	Nil	5 : 1 : 10
	0·05	0·05	0·5	1/240	Nil	Nil	1 : 1 : 10
	0·01	0·05	0·5	1/240	Nil	Nil	0·2 : 1 : 10
	0·005	0·05	0·5	1/180	Nil	Nil	0·1 ; 1 : 10

mentary powers until the ratio content of lecithin was less than approximately 1·5 of lecithin to 1 of cardiolipin to 4–10 of cholesterol.

The Group 4 antigens (cardiolipin content 0·05 per cent.) when tested, show that even so small a proportion of lecithin as 0·1 part to 1 of cardiolipin to 10 of cholesterol is sufficient to suppress any anticomplementary activity of the antigens.

This part of the investigation can be summarized as follows :

(1) When lecithin is tested it has no anticomplementary power.

(2) Mixtures of lecithin with either cardiolipin or cholesterol have no anticomplementary properties.

(3) Under the conditions shown in Table III, the anticomplementary power of cardiolipin Wassermann antigens is completely suppressed when the lecithin content of the antigen is present at a proportion of 1·5 or more parts of lecithin to 1 of cardiolipin to 4–10 of cholesterol.

(4) When cardiolipin Wassermann antigens contain a constant amount of 0·05 per cent. cardiolipin and 0·5 per cent. cholesterol, very small amounts of lecithin (0·005 per cent.) will eliminate the anticomplementary action of the antigen.

It would appear, therefore, that the anticomplementary action of the cardiolipin-cholesterol moiety of cardiolipin Wassermann antigens is lessened by the presence of lecithin in the antigens.

The neutralizing of the anticomplementary power of cardiolipin Wassermann antigens by normal serum is evident.

ANTICOMPLEMENTARY ACTION OF CHOLESTEROL.—In testing the cholesterol component of the cardiolipin Wassermann antigen for anticomplementary action, a range of 1 to 0·125 per cent. in saline suspensions of 1 in 50 and 1 in 100 was employed. No anticomplementary action was discovered in the presence or absence of normal serum.

Next, mixtures of cholesterol and cardiolipin were tested. The range of cholesterol employed varied from 1 to 0·125 per cent., and to each amount was added a constant dose of 0·15 per cent. cardiolipin.

Table IV reveals that :

(1) The mixtures of cholesterol and cardiolipin, when tested in the *absence* of normal serum, show marked destruction of the complement.

(2) The same mixtures, when tested in the *presence* of normal serum, show that the complement has been effectively protected. This protection is almost complete when the mixture contains 0·5 per cent. or less of cholesterol.

Similar tests were also carried out using mixtures containing a like range of cholesterol, but a constant cardiolipin content of 0·05 per cent. The serological pattern obtained was akin to that depicted in Table IV, but, as might be expected, the anticomplementary action was not so marked because of the smaller amount of cardiolipin present in the mixtures.

Mixtures of cholesterol with lecithin were then tested. Two ranges were used, one containing varying amounts of cholesterol from 1 to 0·125 per cent., each unit having a lecithin content of 1·7 per cent. The other consisted of a similar cholesterol range with a constant lecithin content of 0·17 per cent. One part of each of these mixtures was suspended in 200 parts of saline. No anticomplementary properties were demonstrated either in the presence or absence of normal serum.

Finally, cardiolipin Wassermann antigens, containing fixed amounts of cardiolipin and lecithin (both 0·05 per cent.) but varying amounts of cholesterol, from 1·0 to 0·1 per cent., were tested.

TABLE IV

ANTICOMPLEMENTARY EFFECT OF INCREASING AMOUNTS OF CHOLESTEROL IN CHOLESTEROL-CARDIOLIPIN MIXTURES

Mixture (percent.)		Suspension	Normal Serum	Complement Dilutions							
Cholesterol	Cardiolipin			10	20	30	40	50	60	70	80
1·0	0·15	1/200	Absent	−	±	+	+	+	+	+	+
0·75	0·15	1/200		−	±	+	+	+	+	+	+
0·5	0·15	1/200		−	±	+	+	+	+	+	+
0·25	0·15	1/200		−	±	+	+	+	+	+	+
0·125	0·15	1/200		−	−	±	+	+	+	+	+
1·0	0·15	1/200	Present	−	−	−	−	−	+	±	±
0·75	0·15	1/200		−	−	−	−	−	±	±	±
0·5	0·15	1/200		−	−	−	−	−	−	±	±
0·25	0·15	1/200		−	−	−	−	−	−	±	±
0·125	0·15	1/200		−	−	−	−	−	−	−	±
Complement + Normal Saline				−	−	−	−	−	−	−	±
Complement + Normal Serum				−	−	−	−	−	−	−	±

Table V shows that antigens containing a constant amount of cardiolipin and lecithin (0·05 per cent.) become correspondingly more anticomplementary as the amount of cholesterol increases above 0·5 per cent. It has been found that this anticomplementary action of cholesterol-cardiolipin mixtures is limited by the amounts of lecithin present in the antigen. Normal serum is shown to exert a similar but greater effect.

TABLE V

ANTICOMPLEMENTARY EFFECT OF INCREASING AMOUNTS OF CHOLESTEROL IN CARDIOLIPIN ANTIGENS

Antigen Contents (per cent.)			Optimum Titre of Antigen	AC Titre in absence of Normal Serum	AC Titre in presence of Normal Serum	Approximate Ratios of Cholesterol : Cardiolipin : Lecithin
Cholesterol	Cardiolipin	Lecithin				
1	0·05	0·05	1/640	1/40	Nil	20 : 1 : 1
0·8	0·05	0·05	1/480	1/10	Nil	16 : 1 : 1
0·6	0·05	0·05	1/480	1/10	Nil	12 : 1 : 1
0·5	0·05	0·05	1/240	*Nil*	Nil	*10 : 1 : 1*
0·3	0·05	0·05	1/240	Nil	Nil	6 : 1 : 1
0·1	0·05	0·05	1/80	Nil	Nil	2 : 1 : 1

From the work presented on the anticomplementary action of cholesterol, the following points can be made :

(1) Cholesterol itself, when tested in strengths of from 1 to 0·125 per cent. in saline suspensions of 1 in 50 or 1 in 100, shows no evidence of any anticomplementary action.

(2) Mixtures of cholesterol and cardiolipin in saline suspensions of 1 in 200, when tested in the *absence* of normal serum, are markedly anticomplementary if a constant strength of 0·15 per cent. of cardiolipin be used. (see Table IV). If, however, the cardiolipin is kept constant at 0·05 per cent., the anticomplementary property of the mixtures is not quite so pronounced.

(3) If similar mixtures of cholesterol and cardiolipin (constant amounts) are tested in the *presence* of normal serum, the pronounced protection afforded can be demonstrated (see Table IV).

(4) Mixtures of cholesterol and lecithin show no anticomplementary property.

(5) When the three components of cardiolipin Wassermann antigen are mixed, using constant strengths of cardiolipin and lecithin (0·05 per cent.) with increasing doses of cholesterol, the critical anticomplementary point is reached when the ratio of the contents is cholesterol 10, cardiolipin 1, lecithin 1 (see Table V). Up to this point no anticomplementary property can be shown, but if the cholesterol ratio of the antigen be increased, anticomplementary action is evident.

FACTORS AFFECTING THE ANTICOMPLEMENTARY ACTION OF CARDIOLIPIN WASSERMANN ANTIGEN.—As a result of the experiments already performed, the following conclusions can be drawn :

(1) *Within the experimental limits used, the anticomplementary action of cardiolipin Wassermann antigen is due to the effect of the cardiolipin-cholesterol moiety of the mixture because :*

(*a*) cardiolipin, lecithin, and cholesterol, when tested separately, show no anticomplementary properties.

(*b*) cardiolipin + lecithin and cholesterol + lecithin exhibit no anticomplementary properties.

(*c*) cardiolipin and cholesterol mixtures show marked anticomplementary properties (see Tables I and IV).

(2) *Lecithin neutralizes the anticomplementary action of the cardiolipin-cholesterol portion of Wassermann cardiolipin antigens.*

(*a*) In Table I, when 0·05 per cent. cardiolipin was mixed with 0·5 per cent. cholesterol and tested in the absence of normal serum, marked anticomplementary properties were evident. In Table III, when similar strengths of cardiolipin and cholesterol were mixed with 0·05 per cent. lecithin (Group 4 antigens) and then tested, no anticomplementary properties could be demonstrated This neutralizing effect of lecithin only operates within certain well defined limits. Thus, if the cardiolipin content of the above mixture be increased to 0·075 per cent. (Group 2 antigens) and then tested, anticomplementary properties can be demonstrated.

(*b*) In Table III, the neutralizing effect of increasing doses of lecithin present in a series of cardiolipin antigens is shown. It should be noted that in the first three groups of antigens the anticomplementary properties of the antigens, when tested in the absence of normal serum, do not appear until the lecithin content of the antigen falls below approximately 1·5 parts of lecithin to 1 of cardiolipin to 10–4 of cholesterol. In antigens of Group 4, no anticomplementary action was evident, even when the proportion of lecithin was as small as 0·1 of lecithin to 1 of cardiolipin to 10 of cholesterol.

(*c*) The complement protecting power of the lecithin in cardiolipin Wassermann antigens is seen in Table V. The lecithin (0·05 per cent.) present in the antigens (cardiolipin constant at 0·05 per cent.) is capable of dealing with increasing doses of cholesterol until a dose of between 0·5 and 0·6 per cent. is reached. Such antigens, tested in the absence of normal serum, and when containing 0·6 per cent. or more of cholesterol, are anticomplementary.

(3) *Normal serum acts as a powerful protector of the complement when cardiolipin antigens are tested in its presence.*

(*a*) Table I shows the results of testing cardiolipin-cholesterol mixtures for anticomplementary powers both in the presence and in the absence of normal serum.

(*b*) Tables II, III, and V show the power of normal serum to neutralize the anticomplementary effect. None of the antigens when tested in the presence of normal serum show any anticomplementary action. The same Tables show that normal serum is a more powerful protector of complement than lecithin. Clearly, the action of lecithin is restricted, whilst that of normal serum, within the limits shown, is not.

TITRATION OF ANTICOMPLEMENTARY ACTION OF CARDIOLIPIN WASSERMANN ANTIGEN FOR USE IN THE WHITECHAPEL WASSERMANN REACTION TECHNIQUE.—At this stage of the investigation only a tentative formula of the cardiolipin Wassermann antigen for use in the Whitechapel technique can be proposed, because the information so far obtained relates only to its anticomplementary action. From this point of view, the evidence already produced would suggest that the best formula for the cardiolipin antigen should be approximately cardiolipin 1, lecithin 1, cholesterol 10. Thus in Table II, the cardiolipin antigens contain constant amounts of lecithin (0·05 per cent.) and cholesterol (0·5 per cent.), but *increasing amounts of cardiolipin* (from 0·0075 to 0·15 per cent.). On testing, no anticomplementary properties are detected until the proportion of the cardiolipin rises above a ratio of 1 to lecithin 1 and cholesterol 10.

Further evidence in favour of this formula is contained in Table III. Each group of antigens contains constant amounts of cardiolipin and cholesterol, but *increasing doses of lecithin*. In the first three groups, so long as the ratio of the antigen constituents is kept approximately at or above 1·5 parts of lecithin to 1 of cardiolipin to 10–4 of cholesterol, no anticomplementary properties can be demonstrated. This is in spite of the fact that the antigens of Group 3 contain 0·06 per cent. cardiolipin, those of Group 2 contain 0·075 per cent., and those of Group 1 as much as 0·15 per cent. The significant point of this experiment is that the antigens of Group 4 contain constant amounts of 0·05 per cent. cardiolipin and 0·5 per cent. cholesterol, and none exhibits any anticomplementary power. This is so even when the lecithin ratio is as low as 0·1 to cardiolipin 1 and cholesterol 10.

Finally, there is the evidence adduced in Table V. Here the antigens shown contain *increasing doses of cholesterol* with constant amounts of cardiolipin (0·05 per cent.) and lecithin (0·05 per cent.). It will be noted that provided the cholesterol ratio does not rise above 10 to 1 of cardiolipin to 1 of lecithin, no anticomplementary action is demonstrable.

It still remains to be shown what is the best unit ratio strength (as expressed by its percentage composition) of the antigen for use in the Whitechapel technique. It has been demonstrated that if two of the constituents of cardiolipin antigen are kept constant in amount, an alteration of the third constituent must not alter the balance of the antigen ratio content (cardiolipin 1, lecithin 1, cholesterol 10) if anticomplementary action is to be avoided. Thus, if the third constituent be either cardiolipin or cholesterol, it must not be *increased* in amount so as to bring the ratio to lecithin 1, above 1 or 10 respectively (see Tables II and V).

If lecithin be the third component of the antigen to be altered (because its action tends to suppress anticomplementary action of the antigen), this component must not be *decreased* in amount so that its ratio content is below approximately 1 to 1 of cardiolipin and 10 of cholesterol (see Table III).

Nevertheless, it was reasonable to assume that even if the antigen ratio formula of 1 : 1 : 10 were adhered to, if the unit strength were increased sufficiently, a point would be reached where anticomplementary properties would emerge. One indication that this might happen was obtained when cardiolipin was examined. Thus, if 0·3 per cent. or more cardiolipin be suspended in normal saline, no anticomplementary properties can be demonstrated until the strength of the suspension reaches 1 in 50. That the anticomplementary properties of cardiolipin antigen depend not only on a ratio formula, but also on the unit strength of such a formula, is clearly demonstrable by the results of an experiment set out in Table VI.

TABLE VI

ANTICOMPLEMENTARY EFFECT OF INCREASING UNIT STRENGTH OF CARDIOLIPIN WASSERMANN REACTION ANTIGEN FORMULA 1-1-10

Antigen	Per cent. Components			Ratio of Components	Optimum Titre of Antigen	AC Titre in absence of Normal Serum	AC Titre in presence of Normal Serum
	Cardiolipin	Lecithin	Cholesterol				
1	0·15	0·15	1·5	1 : 1 : 10	1/640	1/40	Nil
2	0·075	0·075	0·75	1 : 1 : 10	1/480	1/40	Nil
3	0·05	0·05	0·5	1 : 1 : 10	1/240	Nil	Nil
4	0·025	0·025	0·25	1 : 1 : 10	1/120	Nil	Nil
5	0·0125	0·125	0·0125	1 : 1 : 10	1/90	Nil	Nil

As a result of this experiment it was concluded that if cardiolipin Wassermann antigen for use in the Whitechapel technique be made to the formula cardiolipin 1, lecithin 1, cholesterol 10, at a unit strength of 0·05 per cent., anticomplementary effects would be avoided.

It now remains to integrate these results with those designed to show which formula would produce the most sensitive antigen when used in the Wassermann reaction.

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